The Use of Supercritical Fluid Extraction for the Determination of 4-Deoxynivalenol in Grains: The Effect of the Sample Clean-up and Analytical Methods on Quantitative Results

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Key Words

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Summary

Deoxynivalenol (DON) is one of the trichothecene mycotoxins produced by *Fusarium* molds in grains. Polar cosolvents in supercritical carbon dioxide (SC-CO_2) are needed to extract and isolate the polar DON moiety. This unfortunately results in the extraction of many interfering compounds from the grains into the extracts obtained by supercritical fluid extraction (SFE). Analysis of DON by high performance liquid chromatography (HPLC) using ultraviolet detection (UV) does not provide a specific detection method, although specific detection of DON can be enhanced by using purification steps after SFE. Alternatively, combining SFE with an immunoaffinity method can improve detection specificity and sample cleanup. In this study, SFE was employed to determine DON in grains and cereal products. The effectiveness of the SFE method was compared with two different solvent extraction methods. The extracted DON was quantitatively determined by HPLC-UV using external standardization or competitive enzyme-linked immunosorbent assay (ELISA). In some cases, extracts were purified prior to quantitative analysis of the DON by using solvent partitioning, and/or solid phase extraction, or immunoaffinity columns. Therefore, this paper describes the analysis of DON in cereals using different extraction, cleanup and analysis methods.

Introduction

Mycotoxins, such as deoxynivalenol (DON), are toxicants produced as secondary metabolites from fungi present in grains. The propagation of DON occurs both in the field as well as under storage conditions [1] and can induce toxic effects in certain animal species [2] with potential impact on the health of humans [3]. DON, also known as vomitoxin, has been detected in grain stocks throughout the world, including industrialized nations such as Canada [4], the United States [5–6], Japan [7] and Finland [8–9]. The presence of DON is accentuated during harvest seasons characterized by high humidity, but DON's potential threat to animal and human health requires that routine monitoring be conducted on cereal grains on annual basis [10]. Since DON infestation can occur from sub ppb to ppm levels in several types of grain, analysis methods must be developed that incorporate effective sampling, extraction, and analyte determination protocols.

Several different modern analysis methods have been developed for DON. These have been reviewed by Trucksess et al. for DON in wheat, including high performance liquid chromatography (HPLC) and immunochemical methods [5]. The importance of sample preparation and cleanup prior to chromatographic determination of DON have been noted by Trucksess et al. [6] and Tacke and Casper [11]. Such sample preparation methods employ solid phase extraction (SPE) cartridges before the final analytical determinative method. Recently, immunochemical assays have been developed due to the demand for a rapid method for

Names are necessary to report factually on available data: however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the products to the exclusion of others that may also be suitable.

surveying for DON contamination having relatively high analyte specificity. These have been reviewed by Petska et al.[12] and Chu [13]. More specifically, Park and Chu [14] have reported on immunochemical methods for determining trichothecenes in moldy corn, including the determination of T-2 toxin and aceylated DON by radioimmunoassay and HPLC- enzyme immunoassay.

Analytical supercritical fluid extraction (SFE), which is an alternative for solvent extraction to isolate components from various natural products, has also been applied for mycotoxin analysis [15]. An early study by Kalinoski et al. indicated that ppm level analysis of DON, T-2 toxin, and diacetoxyscirpenol was possible using SFE coupled on-line with mass spectrometry [16]. Selim reported the extraction of aflatoxins and fumonisin utilizing different cosolvent combinations with supercritical carbon dioxide (SC-CO_2) [17]. Recoveries were found to be dependent on the particle diameter of the crushed corn and required an acetonitrile/methanol (2/1) cosolvent mixture to achieve optimal recovery of the aflatoxin with minimal interferring substances. Holcomb et al. reported reasonable recoveries (approx. 80 %) of the major aflatoxins spiked in corn at 3–11 ppb levels using SC-CO_2/methanol mixtures [18].

Taylor et al. systematically investigated the recovery of incurred aflatoxin B_1 in corn and verified the need for cosolvent mixtures to achieve high recoveries [19]. They also attempted to remove coextractives via an in-situ SFE cleanup method and noted that the method was affected by the sample size and heterogeneity of the corn sample. Similarly, Engelhardt and Haas applied SFE for the determination of aflatoxin B_1 in peanut meal and animal feed and found neat SC-CO_2 insufficient for achieving high recoveries of aflatoxin B_1 [20]. Their study indicated a loss in extraction selectivity with increasing cosolvent in SC-CO_2, the latter a requirement for attaining high recoveries of the mycotoxin.

Other sample matrices containing mycotoxins have been treated using SFE. Selim and Tsuei (1993) utilized a CO_2/acetonitrile combination for removing aflatoxin B_1 from grain dusts [21]. Recently Wu et al. [22] and Taylor et al. (1997) [23] used larger quantities of organic cosolvent along with SC-CO_2 at high pressures to remove aflatoxins from peanut meal/kernels and beef liver, respectively. It should be noted that the former study employed an immunoaffinity column for cleanup of the extract, while the latter study achieved excellent recoveries of aflatoxin M_1 at 0.3 ppb level while reducing the quantity of organic solvent required, relative to the established method.

Recently, DON has been successfully extracted from yellow corn meal and rolled oats using methanol as a cosolvent [24]. These investigators indicated that assaying for DON in the extracts by HPLC using ultraviolet (UV) detection did not provide adequate sensitivity for the analyte and was subject to interferences. Similar problems were encountered employing HPLC coupled with mass spectrometry in the full scan mode, although a detection limit of 250 ppb could be achieved using the single ion monitoring (SIM) mode. This study and the others noted above indicate the need for an organic cosolvent coupled with SC-CO_2 to achieve high recoveries of mycotoxins from different sample matrices. Unfortunately this increases the amount of coextracted material which frequently interferes with the subsequent mycotoxin assay methods that are employed. For this reason, several alternative approaches were investigated in the present study in an attempt to overcome these problems.

In this investigation, analytical SFE utilizing SC-CO_2 and cosolvent mixtures were employed for the removal of DON from oat and wheat samples from several sources. Correspondingly, several cleanup methods were evaluated in order to obtain a less complex extract before assaying for DON. These included both the use of traditional sorbents for cleanup as well as employing an immunochemical affinity column. The use of an HPLC-UV method, with and without sample cleanup, as well as commercial enzyme-linked immunosorbent assay kits (ELISA) were also evaluated in this study.

Experimental

Samples

Rolled oats (Quaker Oats, Old Fashioned Brand , The Quaker Oats Company, Chicago, IL, USA) were used for preliminary studies. They were crushed in a mortar and spiked with standard 4-deoxynivalenol prior to SFE. Oats were obtained from Raisio Mills (The Raisio Group, Raisio, Finland, 1995 crop). Prior to extraction, a subsample of 20–50 g was taken and ground using a coffee grinder. The ground oats were again mixed prior to weighing a sample for extraction. When spiked oats were used, 2.4–2.8 µg DON was added into 2–3 g of the ground oats, to produce 1 ppm spike level. Wheat samples from the United States (3 different types) were obtained from Glenn Bennett (NCAUR, USDA-ARS, Peoria, IL, USA). They had previously been homogenized, however they were mixed again thoroughly prior to weighing a sample for extraction.

Reagents/Chemicals

DON (4-deoxynivalenol) was purchased from Sigma Chemical Co. (St. Louis, MO). Granular sodium sulfate, anhydrous was obtained from the Fisher Scientific (Fair Lawn, NJ, USA). Florisil was obtained from Sigma Chemical Co. (St. Louis, MO). Prior to preparation of the cleanup columns, Florisil was dried for 2 hours at 130 °C and kept in a desiccator before it was used. All solvents (methanol, acetonitrile, hexane, trichloromethane) were HPLC-grade from Fisher Scientific (Fair Lawn, NJ, USA). The water that was used in the experiments was obtained from a Milli-Q purification system (Millipore Corp., Bedford, MA).

Liquid Solvent Extraction Methods

Modification of the conventional solvent extraction system described e.g. by Tanaka et al. [7] was used. In this procedure, 25 g of sample was sonicated twice for 5 min after mixing with 100 mL of acetonitrile/water (84/16), and then the mixture was filtered through a Whatman #1 filter paper. A portion of this extract was then defatted using hexane followed by Florisil column cleanup as described by Tanaka et al. [7]. These solvent extracts were then evaporated to dryness and the residue was dissolved in water.

Oat samples were also extracted with water as described in the Veratox® (Neogen Corporation, Lansing, MI) test kit manual as follows: 25 g of oats were extracted with 125 mL of water by vigorously shaking for 3 min. Smaller sample sizes were used for the wheat samples (10 g sample was shaken with 50 mL of water). These extracts were then filtered through a Whatman #1 filter paper. The filtrates were used as such or diluted with water for the ELISA determinations.

Supercritical Fluid Extraction Method

An Isco SFX^{TM} 3560 automated supercritical fluid extraction system (Isco, Lincoln, NE) equipped with two Isco 100DX syringe pumps was used for SFE. Typical sample size was 2–3 g, and the rest of the extraction cell was filled with granulated sodium sulfate. The extraction pressure and temperature were 370 bar and 60 °C, respectively. The flow rate was set at 1.2 mL min^{-1} measured as liquid at the head of the syringe pumps. The automated heated restrictor was held at a temperature of 70 °C.

The SFE method consisted of 7–8 min dynamic mode, followed by a 30 min static hold mode and then a 30 min dynamic mode. The extracts were collected in acetonitrile (5 mL), maintained at 18 °C via liquid CO_2 cooling. Oat flakes spiked with DON were extracted by SFE with selected modifiers in different proportions (V/V). The amount of coextracted components was ascertained from HPLC chromatograms. Eventually, methanol was replaced with a mixture of acetonitrile/water (84/16) as the cosolvent at a 15 % (V/V) level in the SC-CO_2. This was required since the amount of coextracted interferences in the oat extracts made quantification of the DON difficult using the HPLC-UV-system. Coextracted hexane-soluble fatty components were measured gravimetrically in the resultant extracts. The amount of DON extracted was also measured with ELISA as described below.

Extracts from SFE were kept in a freezer at –20 °C for at least two hours to allow the precipitation of the coextracted fat, before the extract was transferred to clean screw-cap tubes. After evaporation of the collection solvent, the extracts were redissolved into water. A portion of this water solution was then used for ELISA, while another portion was taken for affinity column cleanup, or Florisil column cleanup. Such a procedure allowed the use of the two different cleanup and analysis methods for each extract.

HPLC-Method

The HPLC system consisted of a Waters 600E multisolvent delivery system (Waters Corp., Milford, MA) and a Waters 490 multiple wavelength ultraviolet detector. A Hewlett Packard Model 3396A integrator (Hewlett Packard Company, Wilmington, DE) recorded peak areas obtained at 222 nm. The analyses were performed using an Alltech Econosphere C18 (250 × 4.6 mm, 5 µm) column (Alltech Associates, Deerfield, IL). Solvent mixtures of methanol/acetonitrile/water (25/5/75) at a flow rate of 0.6 mL min^{-1}, or methanol/water (25/75) at a flow rate 0.5 mL min^{-1}, were used as mobile phases. To obtain reproducible results, about 15–20 ng of DON, or 20–40 µL of sample, were injected onto the column. This allowed the detection of 0.1 µg g^{-1} (ppm) of DON, but sometimes interferences in the extracts limited the quantification limit to 0.5 µg g^{-1} (ppm).

Immunochemical Materials and Methods

Veratox® (Neogen Corporation, Lansing, MI) quantitative vomitoxin assay kits were used for ELISA determinations. All samples in organic solvents were evaporated into dryness and redissolved in water prior to the ELISA assay. A Bio-Tek Instruments EL308 Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) was used for the measurement of optical density in the test wells. The quantitative linear range of the assay was found to be 0.1–1.2 µg mL^{-1} of DON in the sample solution. This is equivalent to a detection limit of about 15–20 ng g^{-1} (ppb) of DON in the extracts obtained by SFE. This is an improvement over the detection limit of 0.5 µg g^{-1} (ppm) quoted in the test kits when the manufacturer's instructions for sample preparation are used.

Immunoaffinity column purification was achieved using Vicam DONtest^{TM} affinity columns (Vicam Science and Technology, Watertown, MA). This cleanup step was performed as follows: samples in organic solvents were evaporated to dryness and redissolved in water. A portion of the aqueous sample was then introduced into the column by pressure from a glass pipette/hand pump assembly. The affinity column was then washed with water (5 mL) and DON was eluted with methanol (5 mL). Eluates were evaporated to dryness and redissolved in the HPLC mobile phase or water.

Results and Discussion

The major objective of this study was to apply SFE for the analysis of DON in cereal grains, thereby decreasing the overall analysis time and complexity, the number of procedural steps required, and the volume and type of solvents traditionally used. Hence, when coupled with

ELISA, the SFE procedure could have the possibility of implementation in the field. Despite efforts to decrease the amount of coextactives in the extracts, considerable difficulty was encountered with interferences during the HPLC/UV assay for DON in oats. No cosolvent combination with SC-CO_2 proved adequate for minimizing the number of UV-detectable interferences in the HPLC without affecting DON recovery. Similar problems were also encountered by Dragacci et al. using SPE columns and affinity columns, for the HPLC determination of aflatoxin M_1 in cheese products [25]. The number and type of interferences after the SPE cleanup were dependent on the choice of elution solvent in this case. The combination of affinity columns with HPLC eventually allowed them to detect the mycotoxin at 0.2–0.8 ppb levels at 71–88 % recovery levels [25].

Extract Cleanup

Two cleanup methods were used prior to HPLC determination of DON: Florisil and an affinity columns. Typical HPLC chromatograms of the supercritical fluid extracts of ground wheat are shown in Figure 1. Here, the elution position of DON is marked by an arrow on the chromatogram. It is obvious from Figure 1A, that the defatted supercritical fluid extract has many interferences that complicate the HPLC-UV determination. The results from the Florisil column cleanup (Figure 1B) show the retention of several components that could interfere with the assay for DON. Florisil cleanup still permits quantitation of the DON to be accomplished, but it, subject to error using just the one sorbent for cleanup. Although not investigated in this study, multiple sorbent beds employing different sorbents may allow further resolution (in the HPLC analysis) between the interferring compounds and the target analyte [26–28].

After affinity column cleanup of the same extract, only a single peak representing DON appears in the chromatographic profile (Figure 1C), due to its selective binding by the antibodies on the affinity column. This combination of SFE-affinity column-HPLC/UV results in a chromatogram free from UV sensitive interferences. Unfortunately, the relatively low analyte capacity of the affinity columns can cause analysis problems. This manifests itself when analyzing samples having high DON contamination, since the "excess" DON cannot be bound to the column, and hence may be lost during the loading and washing steps.

Table I presents the concentrations of DON in three wheat samples obtained by SFE with subsequent cleanup via Florisil or affinity columns prior to HPLC/UV analysis. There are obviously differences between the SFE results using either Florisil or affinity column cleanup, which are most likely due to the coelution of other extracted moieties from the grains. These could only be successfully separated from DON by use of an affinity column. Supporting the above observation is the fact that the HPLC/UV results from affinity columns

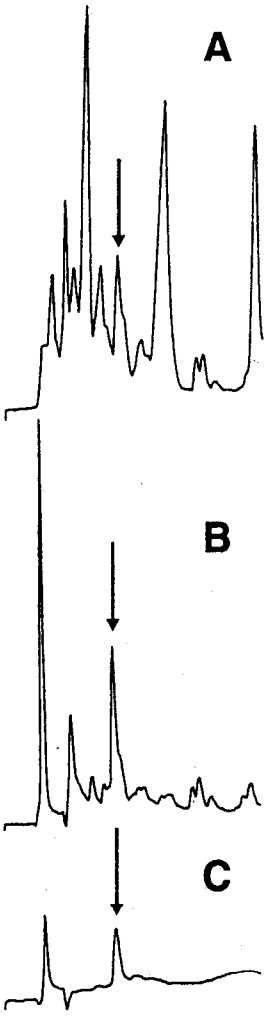

Figure 1
Typical HPLC-UV-chromatograms of the supercritical fluid extracted of ground wheat. DON elution position marked by arrow: (A) defatted extract, (B) Florisil cleaned up extract, (C) affinity column cleaned up extract

are closer to those obtained via ELISA detection for the various wheat and oat samples. The higher error inherent in the SFE-affinity column-HPLC/UV results is probably due to the fact that a much smaller portion of extract that is applied onto affinity column due to its limited sample capacity. This leads to less solute being injected onto the HPLC-system, which increases the corresponding relative standard deviation (RSD) associated with this cleanup and analytical method. Albert and Horwitz [29] estimated that a typical RSD associated with measurement of a 10 or 1 ppb level of analyte is typically 32 and 45 %, respectively, based on the Horwitz exponential relationship between intralaboratory RSD and analyte concentration. Viewed in this context, the results in Table I may not seem so poor.

DON concentration in oats was also determined by HPLC employing the various cleanup methods and ELISA. Again, coeluting components interfered in the HPLC assay, as well as in spiked oat samples. Only affinity columns proved effective for the cleanup of the oat

Table I. Comparison of different cleanup and analysis methods after SFE (n = 3) of DON from oats and wheat samples.

	HPLC-UV (n = 3) Florisil		HPLC-UV (n = 3) affinity		ELISA (n = 3)	
Sample	ave.	%RSD	ave.	%RSD	ave.	%RSD
Wheat1	8.0*	18	3.0	63	5.0	76
Wheat2	10.1	5	9.0	24	8.0	54
Wheat3	4.1	18	3.0	52	1.9	25
Oats	n.d.		n.d.		0.10	42

* ppm ($\mu g\, g^{-1}$)
n.d. = none detected

Table II. Comparison of different cleanup and analysis methods for DON after SFE of oats.

	ELISA defatted	ELISA Florisil	ELISA affinity	HPLC affinity
Spiked[a]				
Rec %	104	79	52	69.8
% RSD	18	19	3.4	16
	n = 5	n = 2	n = 2	n = 2
Natural[b]				
ppm	0.046	0.035	0.038	n.d.
% RSD	29	57	13	
	n = 3	n = 3	n = 3	

[a] results as % recovery at 1 ppm level
[b] results as $\mu g\, g^{-1}$ (ppm)
n.d. = none detected

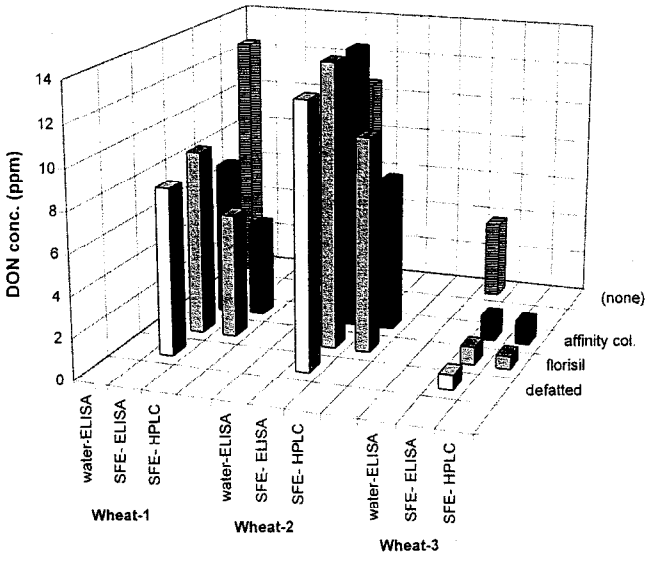

Figure 2
Comparison of analysis results as a function of extraction and analytical method.

extracts for HPLC/UV determination, thus rendering a comparison of the various cleanup methods impossible.

Application of ELISA to Extracts

In addition to the chromatographic method, a competitive enzyme-linked immunosorbent assay (ELISA) was also used to detect DON. The principles underlying ELISA determinations can be found in several references [30–34], and we shall not discuss them here. Results for the SFE using different cleanup steps prior to ELISA assays are presented in the Table II for both spiked and natural oat samples, and for wheat samples as shown in Figure 2. For oats spiked with DON, there is a decreasing recovery of DON for all of the assays proceeded by a cleanup method, relative to simply defatting the sample as described in the experimental section. Whereas no rationale can be offered for this trend, the associated RSDs for the various methods are quite respectable considering the level of analyte concentration.

One possibility for the differences in recorded recoveries in Table II may be due to irreproducibility in the spiking technique or in the cleanup methods. In addition, although the cleanup/ELISA techniques show less than 100 % analyte recovery, they all have utility as qualitative screening methods for DON contamination in oats.

By contrast, DON levels in the extracts of the native oats using different cleanup methods with ELISA detection are quite close to each other. The RSDs associated with these determinations (Table II) are consistent with the Horwitz criterion for the determined DON levels. These are higher than the RSDs for the spiked oats in Table II since they are at concentrations between ten-fold and hundred-fold less in the oat matrix.

Higher RSDs are observed (Tables I and II) in wheat containing higher levels of DON contamination, than in spiked or non-spiked oats having lower contamination levels. This observed difference may be due to the dif-

ferences in extraction efficiency between the two different grain matrices. Alternatively, the high level of DON contamination requires more extensive dilution of extract solutions prior to ELISA analysis, thereby increasing the uncertainity in the final analysis result. For grains contaminated with higher levels of DON (Table I), it was observed that ELISA determination and extracts cleaned up by affinity chromatography followed by subsequent HPLC analysis, showed higher RSDs than those associated with the Florisil-purified HPLC analyzed extracts. This may be due to overloading the affinity column with an analyte concentration that exceeds its binding capacity, i.e., the Florisil cleanup system allows higher analyte concentrations than the affinity column system.

DON concentrations in wheat extracts obtained by water extraction and SFE are shown in Figure 2. In this case, the same supercritical fluid extract was divided into portions and cleaned up by either solvent defatting, or using Florisil, or affinity columns. In Figure 2, the results from the water extraction using the ELISA protocol described in the Veratox® kits are presented using striped columns. For two of the three wheat samples, DON concentrations obtained by extraction with water followed by ELISA were lower than those obtained via SFE/ELISA.

For the wheat samples, ELISA assays gave higher concentration levels of DON in the samples than HPLC-UV, when the same sample cleanup method was applied after SFE (Figure 2). This is consistent with results from previous DON immunoassays, in which the ELISA assays result in the detection of higher mycotoxin levels than the corresponding chromatographic determinations [32–34]. Our results (Figure 2) are similar to those reported recently by Wolf-Hall and Bullerman for DON in wheats [34]. They found higher concentrations of DON in several samples by ELISA assay than by thin-layer or gas chromatography. They indicated that the observed differences between ELISA and chromatographic determination were due to diffferences in analyte recovery from the extraction or cleanup method. For example, water extraction of DON from ground wheat spiked with 1 or 5 ppm, were 83 % and 69 %, respectively, as determined by ELISA. However using organic solvent extraction followed by extract cleanup prior to thin layer chromatography (TLC), yielded only 32 and 26 % recoveries.

It is interesting to note, that in Figure 2, there is consistency between the results obtained by the SFE-ELISA method, regardless of the cleanup method used, for all three wheat samples. This lends some credence to the method, particularly since the DON levels in the different wheat samples were at three different contamination levels. Likewise, results obtained via SFE-HPLC/UV method are also similar whether Florisil or affinity column cleanup was applied. They also appear to be within ± 2 ppm of those results obtained with the SFE-ELISA method.

In a separate study conducted by Bennett et al. on the above wheat samples, higher DON contamination was found than in our study [35]. In their investigation, organic solvent extraction was used with gas chromatographic determination of DON as heptafluorobutyrate derivative. Our reported water extraction combined with ELISA determination yielded only about 50 % of the analyte recoveries compared to Bennett et al.. However, the SFE-ELISA results on the least contaminated sample (Wheat #3) were almost identical to their values (99 ± 9.5) %). The two wheat samples containing higher levels of DON contamination yielded recoveries of 67.8 ± 2.9 % and 33.2 ±3.0 % using the SFE-ELISA method, the latter result being associated with the sample having the highest DON contamination (Wheat #2). This result suggests that the extraction efficiency was decreased with increasing DON contamination level. Similar trends can also be seen in HPLC/UV results in Figure 2.

Conclusions

The techniques and methods reported in this study indicate that SFE can be combined with the proper cleanup or enzyme immunoassay for the selective detection of DON at low levels in grain samples. Overall SFE recoveries in this study showed a dependence on the contamination level of DON being extracted from the grain matrix. Lower recoveries of DON were observed using SFE versus organic solvent extraction. However, supercritical fluid extracts assayed by ELISA showed high recoveries and were accurate relative to DON values determined by other methods. It should also be noted, that the extracts obtained by solvent extraction needed additional cleanup relative to those obtained on an equivalent sample using SFE. This suggests that somewhat better analyte selectivity can be achieved with SFE. It was also observed that the type of cleanup method selected can have an effect on the level of DON detected in a grain sample.

Many of the RSDs in this study appear to be somewhat high by normal standards, but the determined values for DON ranging from 10 ppm to under 0.5 ppb yielded RSDs that are typical of what would be expected when conducting trace toxicant analysis. For example (see Table II), analytes determined at the 1 ppm level typically have a 16 % RSD associated with their measurement, while analysis of 1 or 0.1 ppb DON would be expected to have a RSD of 45 and 64 %, respectively. Therefore the uncertainity in the final result is not necessarily due to inadequate extraction via SFE. Some caution should be exercised however in conducting SFE method development at such low analyte levels, due to the low precision levels that can be attained. It is difficult at these low levels to pinpoint the exact cause of uncertainity in the final analytical result. However, it is also more pertinent to extract incurred residues at these levels, since it is a more accurate reflection of the extraction efficacy and

the final analytical precision that can be expected. In this light, our results are very similar to those found for the SFE of aflatoxin B_1 as reported by Engelhardt and Haas [20].

The HPLC-UV method used in this study was less sensitive than the ELISA assay, although HPLC-UV was capable of detecting DON concentration levels adequate for regulatory monitoring purposes, even at the low levels extracted in this study. Both final detection methods are somewhat affected by the presence of coextracted lipid matter from the grains, no matter what extraction method or grain sample that was being analyzed. Certainly the methods reported in this study can be used for screening purposes, even for the ultra low level detection of DON in grains and similar food products.

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